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Fate of *Salmonella enterica* Serovar Typhimurium on Carrots and Radishes Grown in Fields Treated with Contaminated Manure Composts or Irrigation Water

Mahbub Islam,[1] Jennie Morgan,[1] Michael P. Doyle,[1]* Sharad C. Phatak,[2] Patricia Millner,[3] and Xiuping Jiang[4]

Center for Food Safety, The University of Georgia, Griffin, Georgia 30223[1]; Department of Horticulture, The University of Georgia, Coastal Plain Experiment Station, Tifton, Georgia 31793[2]; Animal Waste Pathogens Laboratory, Beltsville Agricultural Research Service, USDA Agricultural Research Service, Beltsville, Maryland 20705[3]; and Department of Food Science and Human Nutrition, Clemson University, Clemson, South Carolina 29634[4]

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Three different types of compost, PM-5 (poultry manure compost), 338 (dairy cattle manure compost), and NVIRO-4 (alkaline-pH-stabilized dairy cattle manure compost), and irrigation water were inoculated with an avirulent strain of *Salmonella enterica* serovar Typhimurium at 10^7 CFU g^{-1} and 10^5 CFU ml^{-1}, respectively, to determine the persistence of salmonellae in soils containing these composts, in irrigation water, and also on carrots and radishes grown in these contaminated soils. A split-plot block design plan was used for each crop, with five treatments (one without compost, three with each of the three composts, and one without compost but with contaminated water applied) and five replicates for a total of 25 plots for each crop, with each plot measuring 1.8 × 4.6 m. Salmonellae persisted for an extended period of time, with the bacteria surviving in soil samples for 203 to 231 days, and were detected after seeds were sown for 84 and 203 days on radishes and carrots, respectively. *Salmonella* survival was greatest in soil amended with poultry compost and least in soil containing alkaline-pH-stabilized dairy cattle manure compost. Survival profiles of *Salmonella* on vegetables and soil samples contaminated by irrigation water were similar to those observed when contamination occurred through compost. Hence, both contaminated manure compost and irrigation water can play an important role in contaminating soil and root vegetables with salmonellae for several months.

Animal wastes in the form of manure are largely recycled to agricultural land as the most economical and environmentally sustainable means of treatment and reuse. These materials have a beneficial fertilizer value (nitrogen-phosphate-potassium) and can help maintain soil quality and fertility. However, animal manures frequently contain enteric pathogenic microorganisms (15) and land spreading can lead to pathogen entry into the food chain. Therefore, controlling the levels of pathogens in animal wastes used on agricultural fields should help to reduce pathogen contamination of soil, surrounding water, and produce grown in these areas. Although manure is an obvious source of pathogens, two additional sources of pathogens that contaminate soil, water, crops, animals, and humans are runoff water from manure fields and irrigation water containing manure (9). Many outbreaks of infection have been associated with water or food, including processed fruits and vegetables, directly or indirectly contaminated with animal manure (1–6, 20). Cross-contamination of produce from manure or improperly composted manure used on the farm can be a source of pathogen contamination preharvest. Although competition with soil microorganisms and adverse environmental conditions can reduce the number of pathogens, there is little information regarding the degree to which these pathogens can survive in manure-amended soils or in soils irrigated with contaminated water. In this study, our objective was to determine the fate of an avirulent strain of *Salmonella enterica* serovar Typhimurium on carrots and radishes and in surrounding soil when manure composts of different types or irrigation water contaminated with salmonellae were applied to soil in fields typical of those used for vegetable production.

Bacterial culture and inoculum preparation. An avirulent strain of *S. enterica* serovar Typhimurium (χ3985 Δcrp-11 Δcya-12) obtained from Roy Curtiss III, Washington University, St. Louis, Mo., was used for the field study. This strain is a serovar Typhimurium deletion mutant lacking adenylate cyclase and cyclic AMP receptor protein, rendering it avirulent yet still immunogenic (7). This strain grows more slowly than wild-type strains but possesses the wild-type ability to attach to, invade, and persist in gut-associated lymphoid tissue. This strain is unable to utilize maltose, a characteristic differentiating it from other salmonellae and *Enterobacteriaceae*. Cells were thawed from a frozen stock culture, streaked onto tryptic soy agar (TSA; Difco Laboratories, Detroit, Mich.), and incubated for 24 h at 37°C. A single colony from the TSA plate was inoculated into 10 ml of tryptic soy broth (Difco Laboratories) and incubated at 37°C for 16 to 18 h with agitation (150 rpm). A 0.5-ml suspension of the isolate was transferred to 100 ml of tryptic soy broth and incubated for 16 to 18 h with agitation (150 rpm). The bacteria were sedimented three times by centrifugation (4,000 × *g* for 20 min each time) and washed in 0.1% peptone water. The cell pellets were suspended in 0.1% peptone water to achieve an optical density at 630 nm of 0.5

* Corresponding author. Mailing address: Center for Food Safety, The University of Georgia, 1109 Experiment St., Griffin, GA 30223-1797. Phone: (770) 228-7284. Fax: (770) 229-3216. E-mail: mdoyle@uga.edu.

(ca. 10^8 CFU ml^{-1}). Cell numbers were determined by plating of the inoculum on TSA plates.

Inoculation of composts and irrigation water and planting of vegetables. Composts used included PM-5 (poultry manure compost), 338 (dairy cattle manure compost), and NVIRO-4 (alkaline-pH-stabilized dairy cattle manure compost), and were provided by Patricia Millner at the USDA Agricultural Research Service, Beltsville, Md. Each of the three poultry or dairy cattle manure composts was inoculated with serovar Typhimurium at 10^7 CFU g^{-1}. Irrigation water was inoculated with 10^5 serovar Typhimurium ml^{-1}. Compost was applied at a rate of 4.5 metric tons/hectare as a strip 1 day before the planting of seeds. Within a few hours after the carrots and radishes were seeded, a one-time treatment of 2 liters of contaminated irrigation water was hand-sprayed onto the soil of each of the five plots. No chemical treatments for weed control were applied to any of the plots. A split-plot block design plan was followed for each crop, with five treatments (one without compost, three with each of three composts, and one without compost but with contaminated water applied) and five replicates, resulting in a total of 25 plots for each crop. Each plot measured 1.8 × 4.6 m. Carrot (Choctaw variety; Solar Seed Co., Eustis, Fla.) and radish (Red Prince variety; Asgrow Seed Company, Kalamazoo, Mich.) seeds were directly sown on the Horticulture Farm of the Coastal Plain Experiment Station of the University of Georgia, Tifton, in mid-October, according to production guidelines of the University of Georgia Cooperative Extension Service (13).

Sample collection and analysis. At selected time intervals, for each plot for each crop, ca. 100 g of soil was aseptically collected with a sterile spoon in a sterile plastic bag from around a randomly selected plant at 2.5 cm below the surface. From each plot, a randomly selected plant was pulled from the soil and collected aseptically in a sterile plastic bag. Only the edible tuber portions of carrots or radishes were collected as plant samples. The samples were transported to the laboratory in a cooler with ice, placed into a walk-in cooler at 4°C within 4 h of collection, and analyzed within 48 h. Each soil sample (10 g) was mixed with 90 ml of 0.1% peptone water in a sterile Whirl-Pak bag and pummeled in a stomacher for 30 s at low speed. Approximately 5 g of each plant (radish or carrot) was mixed with 45 ml of 0.1% peptone water in a sterile Whirl-Pak bag and rinsed by rubbing and vigorously agitating the plant by hand for 30 s. Serovar Typhimurium counts in peptone water of soil samples and in peptone wash water of plant samples were determined. Serial dilutions (1:10) were prepared from each sample using 0.1% peptone water, and 0.1-ml portions of each dilution in duplicate were spread onto MacConkey agar with 1% maltose plates. Plates were incubated at 37°C for 24 h, and colonies of serovar Typhimurium, which were white with a pink center, were counted. Randomly selected colonies that were white with a pink center were confirmed to be *Salmonella* by a latex agglutination test (Oxoid Inc., Ogdensburg, N.Y.). When the pathogen was not detected by direct plating, 1 g of soil or vegetable sample was added to 99 ml of universal preenrichment broth and incubated at 37°C for 24 h with agitation (150 rpm). Serial dilutions (1:10) of cultures were surface plated in duplicate on MacConkey agar with 1% maltose plates, and colonies were confirmed for *Salmonella* according to the procedure described above. The experimental design was a split plot, where the crop was the main plot and the treatment was the subplot. Each treatment was replicated five times, and each sample from a treatment was plated in duplicate at each sampling time. Hence, *Salmonella* results reported for each data point are the means of 10 values. Data were analyzed by the general linear model procedure of the Statistical Analysis System (SAS Inc., Cary, N.C.).

Salmonella serovar Typhimurium survived for at least 203 days in all the amended soil samples on which carrots or radishes were grown (Fig. 1 and 2). However, in soil samples amended with poultry manure compost PM-5 and dairy cattle manure compost 338, survival was up to 231 days. There was considerable variation in *Salmonella* counts at each sampling time; hence, standard deviations for data points were not included. Analysis of carrots grown in amended soils began on day 42 and that of radishes began on day 21, when the vegetables were large enough for sampling. Serovar Typhimurium was detected for 203 days on carrots and for 84 days on radishes after seeds were sown (Fig. 3 and 4). Initial serovar Typhimurium cell numbers ranged from 1.8 to 3.83 $\log_{10}$ CFU g of $carrots^{-1}$ (Fig. 3) and from 1.53 to 2.36 $\log_{10}$ CFU g of $radishes^{-1}$ (Fig. 4) at the initial day of sampling (42 and 21 days, respectively). Serovar Typhimurium cell numbers on carrots declined progressively with time but remained in approximately the same range through 84 days on radishes. At approximate dates when radishes (day 57) and carrots (day 149) were harvestable, serovar Typhimurium counts were 1.0 to 2.5 and 1.0 to 1.2 $\log_{10}$ CFU g^{-1} on the vegetables, respectively. The radishes (91 days) and carrots (231 days) were grown well beyond normal growing cycles to enable determination of the length of time salmonellae could survive in soil under field conditions.

Mineral and nitrate composition and pH values of the three different compost preparations are presented in Table 1. Nitrogen-phosphate-potassium values were highest for dairy cattle manure compost and lowest for alkaline-pH-stabilized dairy cattle manure compost (Table 1). The pH values of composts PM-5, 338, and NVIRO-4 were 8.1, 8.7, and 7.5, respectively (Table 1). Throughout the study period of nearly 250 days, the pH values of the manure compost-amended soil for both carrots and radishes for all of the treatments did not vary greatly, ranging from 6.7 and 8.0. There were no correlations between differences in the nutrient compositions and pH values of the compost and rates of *Salmonella* inactivation in soil or persistence on vegetables. Moisture contents of the soil varied widely, from 1 to 10%, depending on rainfall (data for pH and moisture content are not shown). Harvest data for the two crops revealed that the greatest yields were from the dairy cattle manure-amended soil. On day 149, when the carrots were ready for harvest, the average weight of a carrot grown on dairy cattle manure compost-amended soil was 122 g, whereas the average weight of a carrot grown on poultry manure compost-amended soil or alkaline-pH-stabilized manure compost-amended soil was 108 or 93 g, respectively. Similarly, on day 57, when the radishes were ready for harvest, the average weight of a radish grown on dairy cattle, poultry, or alkaline-pH-stabilized manure compost-amended soil was 52, 47, or 45 g, respectively. These results conform to the nutritional composition of the composts, with dairy cattle manure compost having the greatest nitrate and mineral contents and alkaline-pH-

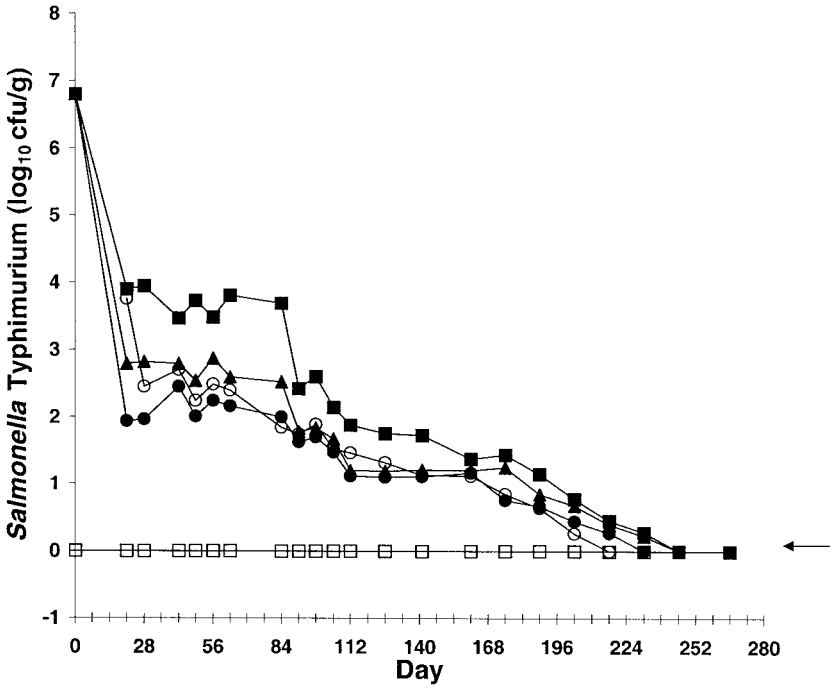

FIG. 1. Survival of *S. enterica* serovar Typhimurium in inoculated-compost-amended or inoculated-water-irrigated soil samples from fields used for growing carrots. Treatments included no compost (□), poultry manure compost (■), dairy cattle manure compost (▲), alkaline-pH-stabilized dairy cattle manure compost (●), and contaminated irrigation water (○). The arrow indicates that the organism was not detectable by enrichment culture.

stabilized dairy cattle manure compost having the least (Table 1).

The avirulent strain of serovar Typhimurium was selected for these studies because of concerns about safe use in the field, where its propagation cannot be controlled. This strain possesses the wild-type ability to attach to, invade, and persist in gut-associated lymphoid tissue. In a similar study that we performed (using a commercial compost and one vegetable

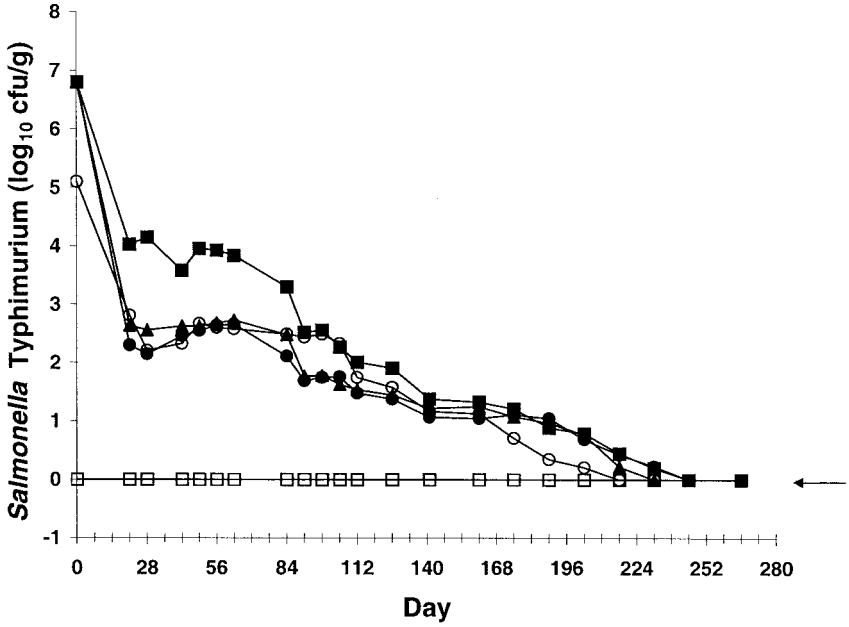

FIG. 2. Survival of serovar Typhimurium in inoculated-compost-amended or inoculated-water-irrigated soil samples from fields used for growing radishes. Treatments included no compost (□), poultry manure compost (■), dairy cattle manure compost (▲), alkaline-pH-stabilized dairy cattle manure compost (●), and contaminated irrigation water (○). The arrow indicates that the organism was not detectable by enrichment culture.

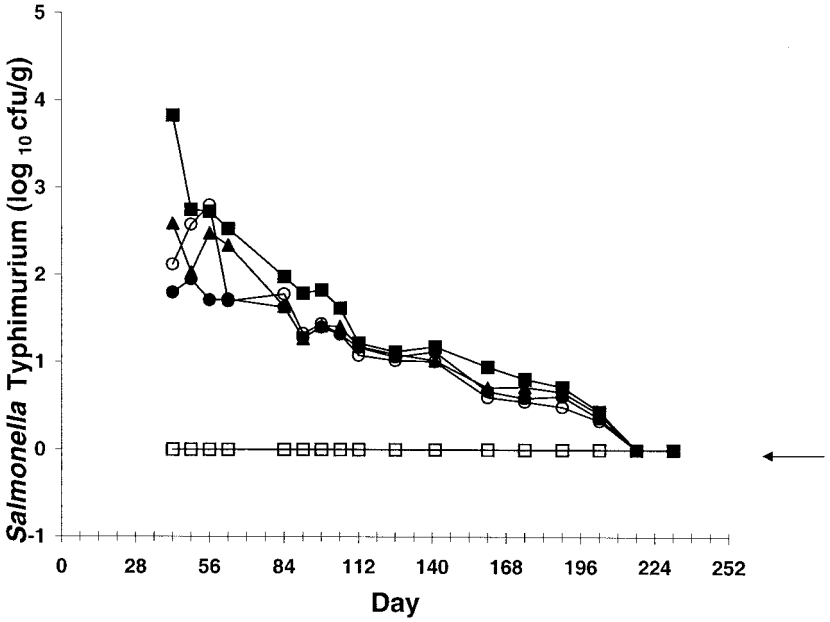

FIG. 3. Survival of serovar Typhimurium on carrots grown in fields containing inoculated-compost-amended or inoculated-water-irrigated soil. Treatments included no compost (□), poultry manure compost (■), dairy cattle manure compost (▲), alkaline-pH-stabilized dairy cattle manure compost (●), and contaminated irrigation water (○). Carrots were harvestable at day 149. The arrow indicates that the organism was not detectable by enrichment culture.

crop) in an environmentally controlled chamber, persistence of this avirulent strain was compared to that of a virulent *S. enterica* serovar Typhimurium strain (ME18). The avirulent mutant strain was detected for 70 days (duration of the study), whereas the virulent strain was not detected after 35 days, suggesting that the avirulent strain is more persistent in soil and on vegetables than the virulent one (unpublished data). *Salmonella* spp. have been reported to survive in soils for long periods of time (up to 968 days) (12). Survival times for up to 300 days in soils spread with cattle slurry have been reported, with survival for up to 259 days having been observed in soils amended with animal feces (12). Factors affecting the survival

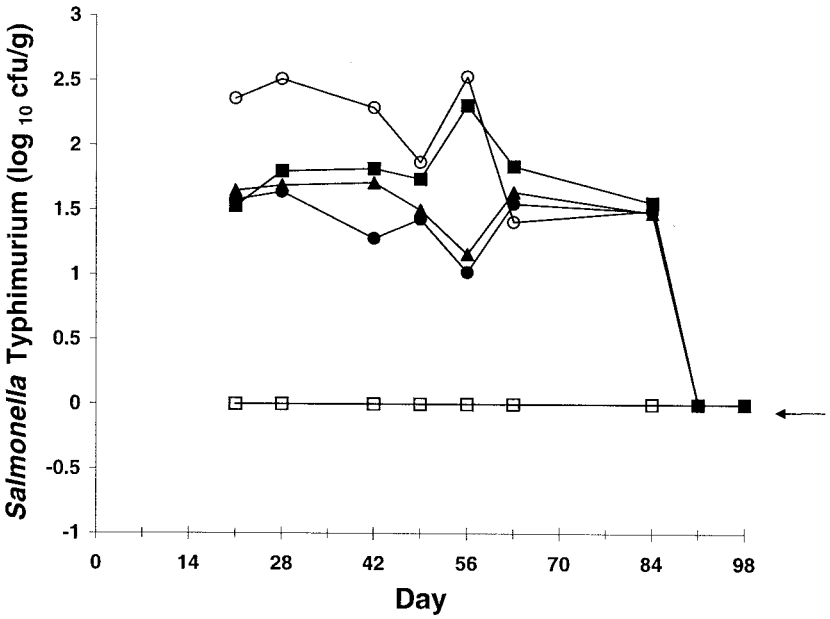

FIG. 4. Survival of serovar Typhimurium on radishes grown in fields containing inoculated-compost-amended or inoculated-water-irrigated soil. Treatments included no compost (□), poultry manure compost (■), dairy cattle manure compost (▲), alkaline-pH-stabilized dairy cattle manure compost (●), and contaminated irrigation water (○). Radishes were harvestable at day 57. The arrow indicates that the organism was not detectable by enrichment culture.

TABLE 1. Mineral and nitrate compositions and pH values of compost preparations[a]

Type of compost	Wt of the indicated mineral (kg/hectare of compost)									NO_3 (ppm)	pH
	Cu	Fe	Mn	Zn	Ca	Mg	K	P	NO_3		
Poultry manure compost PM-5	1.28	3.48	13.29	1.90	5,397	411	16,595	333	1,693	756	8.1
Dairy cattle manure compost 338	12.19	58.25	73.00	31.45	9,203	813	55,688	357	6,989	3,210	8.7
Alkaline-pH-stabilized dairy cattle manure compost NVIRO-4	1.19	3.60	57.75	4.79	8,066	402	3,330	62	185	83	7.5

[a] Values are average of results for four samples.

of *Salmonella* spp. in soil include the initial number of salmonellae, temperature, frost, moisture content, humidity, sunlight, salt concentration, soil texture, organic matter content, and the presence of other microorganisms (11). Several studies have revealed that pathogens applied directly to plants survive for shorter periods of time than those applied to soils (12). In our study, no definitive interpretation of the data could be made relative to the influence of any specific factor on the survival of *Salmonella*.

When manures are applied to land, there is likely to be some movement of the pathogens that they contain through the soil matrix, both vertically and horizontally. The degree of movement will affect the likelihood of pathogens reaching aquifers or surface waters. If these waters are subsequently used for irrigation of produce or for consumption by livestock, there are implications for food safety. Factors known to influence the horizontal movement of pathogens across soils include soil type, soil water content, amount and intensity of rainfall, temperature, nematodal activity, surface charge and size of microorganism, transport through plant roots, and soil pH (14). Factors influencing the vertical movement of pathogens through the soil include the amount and intensity of rainfall, the proximity of the pollutant source, agricultural practice, weather, and the season of application (14, 16). Generally, pathogen survival is favored in aqueous environments, and thus water availability and movement are the single most important factors in determining how far pathogens are likely to move through or across soils. Temperature is also an important consideration, with higher temperatures, e.g., 35°C, reducing pathogen survival (19). Although soil temperatures below the top 5 cm fluctuate seasonally, they are largely unaffected by daily temperature differences. Temperature was determined to be the most important factor influencing pathogen survival in sludge-amended soils, with increasing survival times being a function of decreasing temperature (18, 19). Experiments where sludge was inoculated with *Salmonella* spp. revealed that 45 days was required for a 99% reduction, and persistence times were greater than 5 months (19).

Thermophilic composting is one method for biological stabilization and decomposition of organic substrates in animal manure under conditions which allow for the development of high temperatures (55 to 65°C) resulting from biologically produced heat (17). The final product of composting is sufficiently stable for storage and application to the land without adverse environmental effects. In addition to the stabilization of nutrients, well-managed composting can produce a product that has substantially fewer pathogens than the original manure (10). Bacterial pathogens originally present at levels of 10^4 to 10^6 CFU per g of dry solids can be reduced to undetectable numbers by the end of an efficiently operated composting process (8). However, some composting practices are less effective than others, in part because compost piles are infrequently turned, because moisture content or the pH of compost materials is inadequate for optimal microbial activity, or because there is insufficient ventilation or oxygen content within the compost heaps. Such practices may lead to the survival of substantial numbers of pathogenic bacteria. When reduced turning and temperature monitoring coincide with a situation in which parts of the compostable mass are not exposed to lethal temperatures for a sufficient length of time at the most vulnerable parts of the pile, then pathogens can survive. One of the alternatives to composting as a manure treatment involves the mixing of alkaline by-products, such as fly ash, with manure at high rates to produce an organic lime product in which fecal coliforms, *Escherichia coli*, and *Salmonella* spp. are destroyed within minutes of the mixing of appropriate proportions of the manure and such by-products (P. D. Millner, personal communication, 2002).

Native *Salmonella* spp. were not detected in any of the composts used in our study. With the three composts that were used, the survival of the organism was greatest in soil amended with poultry compost and least in soil amended with alkaline-pH-stabilized dairy cattle manure compost. Studies of alkaline-stabilization treatment of manure have revealed that the normal population of fecal coliforms in manure is reduced by more than 3 $\log_{10}$ CFU g^{-1} within minutes and that there is no increase in fecal coliforms during 4 weeks of subsequent curing (P. D. Millner, personal communication, 2002). The survival rates for *Salmonella* in soil contaminated by irrigation water were similar to those observed for the compost-amended soil. It is remarkable that a one-time application of contaminated irrigation water or compost can result in pathogen contamination of radishes and carrots well beyond their growing cycle. Our results indicate that contaminated irrigation water or manure compost may play an important role in contaminating vegetables and the soil in which they grow.

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